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Abstract: Copper deficiency is a commonly diagnosed problem in cattle around the globe. In Jimma, Ethiopia, 8 zebu (Bos indicus) and 8 zebu × Holstein Friesian cross (Bos taurus × Bos indicus) heifers were used in an 11-wk study to investigate breed type differences and effects of Cu deficiency on concentrations of trace elements in plasma and edible tissues as well as mRNA expression of Cu-related genes. Heifers were fed a grass diet (6.4 ± 0.2 [SEM] mg Cu/kg DM) supplemented with 1 mg Mo/kg DM in wk 1 to 4 and 2 mg Mo/ kg DM in wk 5 to 11, with blood samples collected every 2 wk and tissue collection postmortem. Plasma, liver, kidney, and semitendinosus and cardiac muscle were analyzed for Zn, Cu, Fe, Se, Mo, Co, and Mn. Expression of mRNA Cu-related genes was measured in aorta (lysyl oxidase [LOX]), liver (Cu transporting -polypeptide [Atp7b], Cu chaperone for superoxide dismutase [CCS], cytochrome c oxidase assembly homolog 17 [Cox17], Cu transporter 1 homolog [Ctr1], and superoxide dismutase 1 [Sod1]), and duodenum (diamine oxidase [DAO] and metallo-thionein-1A [Mt1a]) as well as the Se-related glutathione peroxidase 1 (Gpx1). Zebu cattle maintained initial plasma Cu concentrations just below the threshold value for deficiency, whereas crossbred cattle gradually became severely Cu deficient over time ($P < 0.001$). In contrast, plasma Zn and Co were greater in zebu cattle at the onset of the trial but became similar to crossbred cattle towards the end of the trial ($P < 0.001$). Liver Cu ($P = 0.002$) and Fe (P 0.001), kidney Se ($P < 0.001$), and kidney and cardiac muscle Co (P 0.001) concentrations were greater in zebu than in crossbred cattle. Increased hepatic mRNA expression of the Cu regulatory genes Atp7b, Ctr1 ($P = 0.02$), CCS ($P = 0.03$), and Cox17 ($P = 0.009$) and Cu-related Sod1 ($P = 0.001$) as well as the Se-related Gpx1 (P 0.001) were greater in zebu than in crossbred cattle. However, duodenal mRNA expression of DAO ($P = 0.8$) and Mt1a ($P = 0.2$) and aortic expression of LOX ($P = 0.8$) were not different. Both the differences in Cu status indices (plasma and liver concentrations) and hepatic mRNA expression of Cu regulatory genes point to the possibility of a more efficient use of dietary Cu in B. indicus as compared to B. taurus × B. indicus cattle resulting in greater sensitivity to Cu deficiency in B. taurus crossbred cattle.

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A disparate trace element metabolism in zebu (*Bos indicus*) and crossbred (*Bos indicus* × *Bos taurus*) cattle in response to a copper-deficient diet[1]

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ABSTRACT: Copper deficiency is a commonly diagnosed problem in cattle around the globe. In Jimma, Ethiopia, 8 zebu (*Bos indicus*) and 8 zebu × Holstein Friesian cross (*Bos taurus* × *Bos indicus*) heifers were used in an 11-wk study to investigate breed type differences and effects of Cu deficiency on concentrations of trace elements in plasma and edible tissues as well as mRNA expression of Cu-related genes. Heifers were fed a grass diet (6.4 ± 0.2 [SEM] mg Cu/kg DM) supplemented with 1 mg Mo/kg DM in wk 1 to 4 and 2 mg Mo/kg DM in wk 5 to 11, with blood samples collected every 2 wk and tissue collection postmortem. Plasma, liver, kidney, and semitendinosus and cardiac muscle were analyzed for Zn, Cu, Fe, Se, Mo, Co, and Mn. Expression of mRNA Cu-related genes was measured in aorta (lysyl oxidase [*LOX*]), liver (Cu transporting β-polypeptide [*Atp7b*], Cu chaperone for superoxide dismutase [*CCS*], cytochrome c oxidase assembly homolog 17 [*Cox17*], Cu transporter 1 homolog [*Ctr1*], and superoxide dismutase 1 [*Sod1*]), and duodenum (diamine oxidase [*DAO*] and metallo-thionein-1A [*Mt1a*]) as well as the Se-related glutathione peroxidase 1 (*Gpx1*). Zebu cattle maintained initial plasma Cu concentrations just below the threshold value for deficiency, whereas crossbred cattle gradually became severely Cu deficient over time ($P < 0.001$). In contrast, plasma Zn and Co were greater in zebu cattle at the onset of the trial but became similar to crossbred cattle towards the end of the trial ($P < 0.001$). Liver Cu ($P = 0.002$) and Fe ($P \leq 0.001$), kidney Se ($P < 0.001$), and kidney and cardiac muscle Co ($P \leq 0.001$) concentrations were greater in zebu than in crossbred cattle. Increased hepatic mRNA expression of the Cu regulatory genes *Atp7b*, *Ctr1* ($P = 0.02$), *CCS* ($P = 0.03$), and *Cox17* ($P = 0.009$) and Cu-related *Sod1* ($P = 0.001$) as well as the Se-related *Gpx1* ($P \leq 0.001$) were greater in zebu than in crossbred cattle. However, duodenal mRNA expression of *DAO* ($P = 0.8$) and *Mt1a* ($P = 0.2$) and aortic expression of *LOX* ($P = 0.8$) were not different. Both the differences in Cu status indices (plasma and liver concentrations) and hepatic mRNA expression of Cu regulatory genes point to the possibility of a more efficient use of dietary Cu in *B. indicus* as compared to *B. taurus* × *B. indicus* cattle resulting in greater sensitivity to Cu deficiency in *B. taurus* crossbred cattle.

Key words: *Bos indicus*, cattle, copper, deficiency, trace element, zebu

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INTRODUCTION

The dietary micromineral supply for cattle is often low in tropical countries (McDowell and Arthington, 2005). Mineral imbalances in the soil and consequently in the plants growing on this soil often aggravate this situation (Haque et al., 1993). In Ethiopia, copper deficiency is a frequent problem when grazing on pastures, as low levels of Cu are accompanied by high levels of Mo and S and very high levels of Fe (Roeder, 1980; Faye and Grillet, 1984; Faye et al., 1991; Dermauw et al., 2013a). Consequently, deficiencies of Cu are to be expected in cattle in these regions.

Zebu cattle (*Bos indicus*) are the most common type of cattle in Ethiopia and renowned for their robustness under tough conditions (Edea et al., 2013). Moreover, they seem to have adapted to their environment very well, with reports indicating an ability to cope with dietary antagonists, such as tannins (Yisehak et al., 2012). However, because of their low production capacity, crossbreeding these *B. indicus* cattle with *Bos taurus* types is becoming popular (Fekadu et al., 2011). Within *B. taurus* cattle, breed-related sensitivity to Cu deficiency has been documented, with the Simmental breed reported as being most vulnerable (Mullis et al., 2003; Ward et al., 1995). Several studies suggest that there may be a difference between *B. taurus*-influenced and *B. indicus* cattle in their ability to cope with an inadequate Cu supply. Frequently, no signs of clinical copper deficiency are seen in studies with zebu cattle despite severely inadequate Cu supply based on recommended levels for *B. taurus* cattle (Roeder, 1980; Faye et al., 1991; Dermauw et al., 2013b). Other studies suggest that there might be a difference in response to the same diet between the local zebu cattle and *B. taurus* crossbred cattle (Friot, 1973; Damir et al., 1988).

As we hypothesized that *B. indicus* may have adapted to a low Cu intake, hence optimizing the use of Cu in their metabolism, our aim was to evaluate the effect of a Cu-deficient diet on concentrations of Cu and other trace elements in plasma and edible tissues as well as mRNA expression of Cu-related genes in *B. indicus* and *B. taurus* × *B. indicus* cattle.

MATERIALS AND METHODS

Animal Care and Experimental Design

Sixteen *B. indicus* (Abyssinian Highland zebu; n = 8) heifers and *B. taurus* × *B. indicus* crossbred (Holstein Friesian × Abyssinian Highland zebu; n = 8) heifers with an average age of 1 yr and 6 mo (SD = 0.4 yr) were purchased and housed at the dairy farm of the Jimma University Campus of Agriculture and Veterinary Medicine, Ethiopia. In the surrounding area, bovine Cu deficiency was seen on multiple occasions and Se deficiency was suspected based on tissue levels below threshold values for *B. taurus* cattle (Dermauw et al., 2013a,b).

Table 1. Chemical composition of basal grass diet samples (n = 11)

Component	Mean	SEM
CP, % DM	11.5	0.37
ADF, % DM	35.4	0.81
NDF, % DM	67.7	0.59
S, g/kg DM	2.4	0.12
Fe, mg/kg DM	250	27.9
Mn, mg/kg DM	102	15.9
Zn, mg/kg DM	64	3.7
Cu, mg/kg DM	6.4	0.23
Co, mg/kg DM	0.22	0.016
Mo, mg/kg DM[1]	2.1	0.41
Se, mg/kg DM	0.11	0.026

[1]Supplemented with 1.0 mg Mo/kg DM from wk 1 to 4 and with 2.0 mg Mo/kg DM from wk 5 to 11, as $NaMoO_4$.

All animals were individually housed and fed with a forage-only diet, consisting of mature local grasses, freshly harvested and chopped (20 cm), during 11 wk. This diet contained 6.4 ± 0.2 (SEM) mg Cu/kg DM, 2.1 ± 0.4 mg Mo/kg DM, and 2.4 ± 0.12 g S/kg DM (Table 1). Daily amounts of grasses provided were set to provide a DM intake of 2% of BW (McDowell, 1996). Refusals were weighed and subtracted from the offered amount of grass daily. To induce Cu deficiency, Mo (as $NaMoO_4$; Sigma-Aldrich, St. Louis, MO) was supplemented at 1 mg/kg DM during wk 1 to 4 and raised to 2 mg/kg DM from wk 5 to 11. The supplements were mixed in a spoon (15 mL) of molasses and top-dressed on the grass diet.

Every 2 wk, the BCS of heifers was noted. The BCS of crossed heifers was evaluated on a scale of 1 to 5 for *B. taurus* dairy cattle (Wildman et al., 1982), whereas for zebu heifers, the BCS was evaluated using the *B. indicus* scale of 1 to 9 (Nicholson and Butterworth, 1986). Zebu BCS subsequently were recalculated to a scale of 1 to 5, for comparison purposes.

Samples and Storage

Representative feed samples were collected 3 times a week and stored at –40°C. Afterward, samples were pooled per week, oven dried at 65°C until constant weight, and subsequently ground through a 2-mm sieve. Every 2 wk, 9 mL blood was collected from all animals by jugular venipuncture using 18 gauge needles (450069) and 2 sodium heparin tubes (455051; both Vacuette; Greiner Labortechnik, Kremsmunster, Austria). At wk 11, all animals were brought to a local slaughterhouse and slaughtered. Approximately 50 g of liver (caudal lobe), kidney (cranial part of left kidney), and cardiac (heart apex) and

semitendinosus muscle were collected to determine trace element concentrations in these tissues. Additionally, samples of aorta, small intestine, and liver were collected to determine mRNA expression of Cu transporters and Cu-related enzymes.

Plasma was obtained through centrifugation at 1,500 × *g* for 10 min at 25°C and was stored at –20°C until further analysis. Tissue samples for mineral analyses were initially frozen at –40°C, afterward oven dried at 65°C until constant weight, and ground through a 2-mm sieve. Tissue samples for mRNA analyses were immediately immersed in 10 mL RNAlater (Sigma-Aldrich) and frozen at –40°C.

Analytical Procedures

Grass samples were analyzed for CP (method 990.03; AOAC, 2000), ADF (method 973.18; AOAC, 2000), and NDF (Van Soest et al., 1991). Plasma, grasses, and tissue samples for mineral analysis were prepared through microwave digestion with 10 mL HNO_3 (ultrapure analytical grade for trace element analysis) in open vessels followed by filtration through syringe filters (Chromafil RC-45/25, regenerated cellulose, pore size 0.45 μm; Macherey-Nagel, Düren, Germany). All samples were analyzed for Zn, Cu, Fe, Se, Mo, Co, and Mn concentrations and grass samples additionally for S through inductively coupled plasma optical emission spectrometry (Vista MPX radial; Varian, Palo Alto, CA) and inductively coupled plasma mass spectrometry (Elan DRC-e; PerkinElmer, Sunnyvale, CA). All glassware and microwave vessels were prerinsed with diluted HNO_3. A quality control program was used throughout mineral analyses. Recovery rates from sampled matrices, spiked with 2 concentrations of the studied trace elements (in the range of the determined concentrations), were measured. Average recovery was 98%, with a range between 82% (Zn in plasma) and 109% (Mo in kidney). Lower detection limits in acid digest were determined as 0.35 μg/L for Mn, 0.25 μg/L for Cu, 0.33 μg/L for Mo, 0.13 μg/L for Se, 21.4 μg/L for Fe, 16.4 μg/L for Zn, and 0.14 μg/L for Co. Control standards were analyzed after every 20 samples or less followed by a recalibration. The obtained results of each sample batch were only accepted if the deviation of each concentration measured in the control standards from the targeted concentration was less than 5%. All analytical results were blank corrected.

Quantitative Real Time Reverse Transcription PCR

Total RNA was isolated from tissue samples using either the RNeasy Mini Kit (intestine and liver) or RNeasy Fibrous Tissue Mini Kit (aorta; Qiagen Ltd., Manchester, UK). Tissue samples (mean = 48 mg and range = 35 to 60 mg) were homogenized in a TissueLyser (Qiagen Ltd.) using 500 μL of lysis buffer from the extraction kits and a 5-mm steel ball bearing in a 2-mL Safe-Lock tube (Eppendorf, Stevenage, UK), with 3 cycles of shaking at 20 Hz for 2 min. Lysates were then processed as per the manufacturer's instructions, including the optional on-column deoxyribonuclease (**DNase**) digestion step. The RNA was eluted in 2x 50 μL of nuclease-free water. Further DNase digestion of the RNA solution was performed using RQ1 RNase-Free DNase (Promega Ltd., Southampton, UK) as per the manufacturer's instructions with the sample incubated for 30 min at room temperature. To remove the DNase and reaction buffer from the purified RNA, it was passed through the RNeasy Mini Kit using the RNA clean-up protocol and was eluted in 2x 40 μL of elution buffer (10 m*M* Tris HCl, pH 8.4). The RNA concentration in the eluate was measured using the Qubit RNA Assay Kit (Invitrogen, Paisley, Scotland).

Primers and probes (Table 2) were designed using Primer 3 (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu) and M-Fold (SantaLucia, 1998; available at http://mfold.rna.albany.edu/?q=mfold) using the bovine-specific GenBank sequences for the potential housekeeper genes: β-2-microglobulin (*B2M*; NM 173893), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; NM 001034034), hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*; NM 001034035), ribosomal protein S8 (*RPS8*; NM 001025317), succinate dehydrogenase complex, subunit A (*SDHA*; NM 174178), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWAZ*; XM 001788370) as well as adenosine triphosphatase, copper transporting, β polypeptide (*Atp7b*; XM 002691794), copper chaperone for superoxide dismutase (*CCS*; NM 001046187), cytochrome c oxidase assembly homolog 17 (*Cox17*; XM 002684734), copper transport 1 homolog (*Ctr1*; NM 001100381), diamine oxidase (*DAO*; NM 001034361), glutathione peroxidase 1 (*Gpx1*; NM 174076), lysyl oxidase (*LOX*; NM 173932), metallothionein-1A (*Mt1a*; NM 001040492), and superoxide dismutase 1 (*Sod1*; NM 174615) as described previously (Peters et al., 2004). Primer specificity was tested using the Primer BLAST algorithm (www.ncbi.nlm.nih.gov).

Synthesis of cDNA was performed with 500 ng of random hexamers using the ImProm-II Reverse Transcription (**RT**) System (Promega Corp., Madison, WI) using 600 to 1,000 ng (liver and intestine) or 200 ng (aorta) of total RNA in a final volume of 40 μL. All reactions were prepared according to the manufacturer's instructions giving a final magnesium chloride concentration of 3 m*M*.

Reverse transcription was performed in a PTC-200 DNA engine (Bio-Rad Laboratories Inc., Hercules, CA). Duplicate RT reactions were performed for each RNA sample. All cDNA were diluted to a final volume of

Table 2. Real-time RT-PCR primers of Cu regulatory genes examined in aorta, duodenum, and liver of zebu (*Bos indicus*) and crossbreed (*Bos indicus* × *Bos taurus*) heifers on a Cu-deficient diet

Gene	Assay	Forward primer	Reverse primer	PS,[1] bp	E[2]	PMT[3]
Atp7b	Control	TAGAAGGCAAGATCGGGAAA	CTGGGGAGACGAGAGAAGG	435		
	qPCR[4]	TAGAAGGCAAGATCGGGAAA	ATGTGGTCCCTGAGGTCTTG	121	94.9	87.0
B2M	Control	GTTCCATCCACCCCAGATT	TTACAGGTCTCGATCCCACTT	211		
	qPCR	GTTCACTCCCAACAGCAAGG	ACTATCCGGGGTTGTTCCA	72	98.7	82.0
CCS	Control	CTGTGGGGACCACTTTAACC	AGGCCATCACAGGAGCAG	388		
	qPCR	CAGGATCACAGGAAACTCAGG	GCTTGGGGTTCTGGAAGAG	80	96.1	85.5
Cox17	Control	TGAGTCGCAGGAGAAGAAGC	TCAGCAAGGAACTCCCAAAG	233		
	qPCR	GAAAGGAGAAGAGCAATGTGGA	ATTCACTCCCAGAGCAGACC	102	99.1	83.0
Ctr1	Control	TGGGGATGAACATGGATATG	AATGGCAATGCTCTGTGATG	549		
	qPCR	CCCAACCACTTCATCTGACC	AAAGCTCCAGCCATTTCTCC	138	99.3	83.0
DAO	Control	GCACTGTACGGAGGACACAC	CCGTTGGGGTAGAAGATGAA	350		
	qPCR	ACGCCCTCCACTACTACGAC	GGGCATCTCGAAGAGACAGA	71	96.7	86.0
GAPDH	Control	ACCAGGGCTGCTTTTAATTCT	GGTCATAAGTCCCTCCACGA	474		
	qPCR	GGGTCATCATCTCTGCACCT	GGAGGCATTGCTGACAATCT	101	99.6	84.5
Gpx1	Control	AACGTAGCATCGCTCTGAGG	AGCATAAAGTTGGGCTCGAA	203		
	qPCR	CGGGACTACACCCAGATGA	TCCTCGTTCTTGGCGTTT	113	93.5	89.5
HPRT1	Control	CCAGTCAACAGGCGACATAA	GGCTCGTAGTGCAAATGAAGA	527		
	qPCR	GCGAAACTGGAAAAGCAAAA	GCCACAGAACAAGAACATTGG	116	93.0	82.0
LOX	Control	ATACGGCACCGGCTACTTC	CCCTCAGCCACTCTCCTCT	348		
	qPCR	CCCCAGAGAGTGAAAAACCA	TGGCATCAAGCAGGTCATAG	139	95.7	84.0
Mt1a	Control	CTGCTTTGCCACTTGTTCTG	GCACCAGGTCAGATTGTATGAA	342		
	qPCR	CTGATGTCGGGGAGAACCT	AAGGTAATGTAGCACCAGGTCAG	101	98.2	79.0
RPS8	Control	CATCTCTCGGGACAACTGG	TTCGCGTTCTTTTTCCTCTC	464		
	qPCR	CATCTCTCGGGACAACTGG	GCGTCCCAGCTCATACTTTC	88	96.7	86.0
SDHA	Control	TGCAGACCCGGAGATAAAGT	CGTACTCGTCAACCCTCTCC	391		
	qPCR	TAAACCAAATGCTGGGGAAG	CTGCATGTTGAGTCGCAGTT	97	95.1	81.5
Sod1	Control	GCAAGGCACCATCCACTT	CACCTCTGCCCAAGTCATCT	341		
	qPCR	GGATTCCACGTCCATCAGTT	GGTCTCCAACATGCCTCTCT	121	98.4	84.0
YWAZ	Control	GCTTCACAAGCAGAGAGCAA	CCGATGTCCACAATGTCAAG	367		
	qPCR	ACTGGGTCTGGCCCTTAACT	TGGCTTCATCAAATGCTGTC	98	97.9	82.0

[1]PS = product size.

[2]E = efficiency.

[3]PMT = product melting temperature.

[4]qPCR = quantitative PCR.

200 µL (1:5 dilution) using EB Buffer (10 m*M* Tris-HCl, pH 8.4; Qiagen Ltd.) and then stored at –20°C for future use. Nontemplate controls were performed by addition of nuclease-free water in place of RNA.

Quantitative PCR (**qPCR**) was performed using GoTaq Colourless Master Mix (Promega Corp., Madison, WI). Gene specific amplification was performed using 0.2 µ*M* of each primer, SYBR Green 1 (1:100,000 final concentration; Invitrogen), ROX (1:5,000; Invitrogen), and 5 µL of diluted cDNA in a final volume of 25 µL. Magnesium chloride concentrations were adjusted to 4.5 m*M* in the final reaction by addition of 50 m*M* $MgCl_2$.

Sample incubations were performed in an MxPro 3005P (Agilent Technologies, Wokingham, Berkshire, UK) at 95°C for 2 min and then 45 cycles of 95°C for 10 s and 60°C for 30 s during which the fluorescence data were collected. Threshold values (**Ct**) for the samples were calculated using the MxPro qPCR software (version 4.1; Agilent Technologies) using the multiple experiment analyzer with run-to-run variations in Ct normalized using a positive control of known copy number and ROX as a passive reference dye.

The absence of genomic contamination of the RNA samples was confirmed before the RT reactions and none of the samples showed evidence of amplifiable genomic DNA with the SDHA qPCR assay. One qPCR reaction was run for each RT repeat resulting in 2 Ct values for each RNA sample.

To determine the most appropriate housekeeper genes for the study, all 7 potential genes were quantified in 6 cDNA samples from each tissue type. A mean Ct value was calculated for each sample using the 2 measured Ct values for each sample for each of the potential housekeeper genes. The mean Ct value was converted to a relative copy number

value using the $E^{\Delta Ct}$ method (E = reaction efficiency as determined from a standard curve) with ΔCt values calculated relative to the sample with the largest Ct (fewest gene copies). The geNorm VBA applet (Vandesompele et al.,2002; available at http://medgen.ugent.be/%7Ejvdesomp/genorm/) for Microsoft Excel (Microsoft Corp., Redmond, WA) was used to determine the most stable genes from the set of tested genes. The 3 most stable housekeeper genes for the aorta samples were *B2M*, *HPRT1*, and *SDHA*; for the intestine were *GAPDH*, *SDHA*, and *YWAZ*; and for the liver were *B2M*, *HPRT1*, and *SDHA*. The 3 selected housekeeper genes were then quantified in the remaining samples and these genes were then used to normalize the results for the other genes quantified.

A relative copy number was calculated for each sample using the qBase applet for Microsoft Excel (http://medgen.ugent.be/qbase) using the 3 housekeeper genes to normalize the results, using the methods described by Vandesompele et al. (2002). The sample with the fewest gene copies (latest Ct value) is given a relative copy number of 1 and all other samples are given values relative to this sample. This relative copy number result was used for all comparisons involving mRNA expression.

To assess reaction efficiency, a set of primers was designed for each gene target to amplify a larger fragment, which included the portion amplified by the qPCR assay. These assays were tested against a cDNA obtained from RNA extracted from each of the tissues. Products were separated by 2% agarose gel electrophoresis, purified by NucleoSpin Extract II kit (Macherey-Nagel), and then quantified using the Qubit dsDNA BR Assay (Invitrogen). The number of copies per microliter of purified product was calculated and then a 1:10 dilution series from 10^7 to 1 copy per qPCR was analyzed in triplicate using the qPCR assay and the reaction efficiency calculated using the MxPro software.

Statistical Analyses

All statistical procedures were performed using the mixed model (SAS Inst. Inc., Cary, NC). For weight, BCS, and plasma mineral concentrations, the model included sampling week as a continuous fixed effect, breed type as categorical fixed effect, and their interaction. Heifer was considered as the experimental unit and was inserted as a random effect. For tissue mineral concentrations, the model included tissue and breed type as a categorical fixed effect and their interaction. Again, heifer was incorporated as a random effect. To compare mRNA expression and performance data, a fixed effects model was used with type included as categorical fixed effect. Associations between trace element storage and mRNA expression were evaluated using Spearman correlation tests.

RESULTS

Plasma Mineral Concentrations

Plasma Cu concentrations changed differently over time in the 2 breed types (breed type × week interaction; $P < 0.001$; Fig. 1), with concentrations in crossbred heifers decreasing more dramatically than in zebu heifers. Overall, plasma Cu concentrations decreased throughout the trial (week; $P < 0.001$). Plasma concentrations of both Fe and Mo were not affected by breed type (breed type × week; $P = 0.8$, $P = 0.3$, respectively). Over time, plasma Mo increased (week; $P = 0.04$), whereas plasma Fe concentrations decreased (week; $P < 0.001$).

Plasma Zn concentrations changed differently over time between the 2 breed types (breed type × week; $P = 0.04$). However, unlike in the case of Cu, plasma Zn concentrations seemed to differ between breed types at the start of the trial with a diminishing difference throughout the trial. Overall, plasma Zn concentrations were also affected by a breed type effect (breed type; $P = 0.001$), with lower concentrations in crossbreeds than in zebu heifers throughout the trial. Over time, plasma Zn concentrations decreased (week; $P < 0.001$).

Plasma Mn concentrations were not affected by breed type (breed type × week; all $P = 0.3$) but tended to decrease slightly over time (week; $P = 0.05$). Throughout the trial, plasma Se concentrations increased (week; $P < 0.001$) but did not differ between breed types (breed type × week; $P = 0.2$). Over time, plasma Co concentrations changed differently in both breed types (breed type × week; $P = 0.001$), where the difference between breed types seemed large at the onset of the trial but afterward a smaller difference remained constant over time. Plasma Co concentrations were also subjected to an overall breed effect throughout the trial (breed type; $P < 0.001$). Again, concentrations in crossbreeds were lower than in zebu heifers. Throughout the trial, plasma Co concentrations decreased (week; $P < 0.001$).

Tissue Mineral Concentrations

The relative Cu tissue concentrations in the different organs tended to be different in the 2 breed types (breed type × tissue interaction; $P = 0.06$; Fig. 2). Liver Cu concentrations were greater in zebu than in crossbred heifers ($P = 0.002$). No differences were found for other tissues (cardiac muscle, $P = 1.0$, and kidney and semitendinsosus muscle, $P = 0.9$). Relative tissue Mo concentrations were not impacted by breed type (breed type × tissue; $P = 1.0$). On the contrary, relative tissue concentrations of Fe were different in the 2 breed types

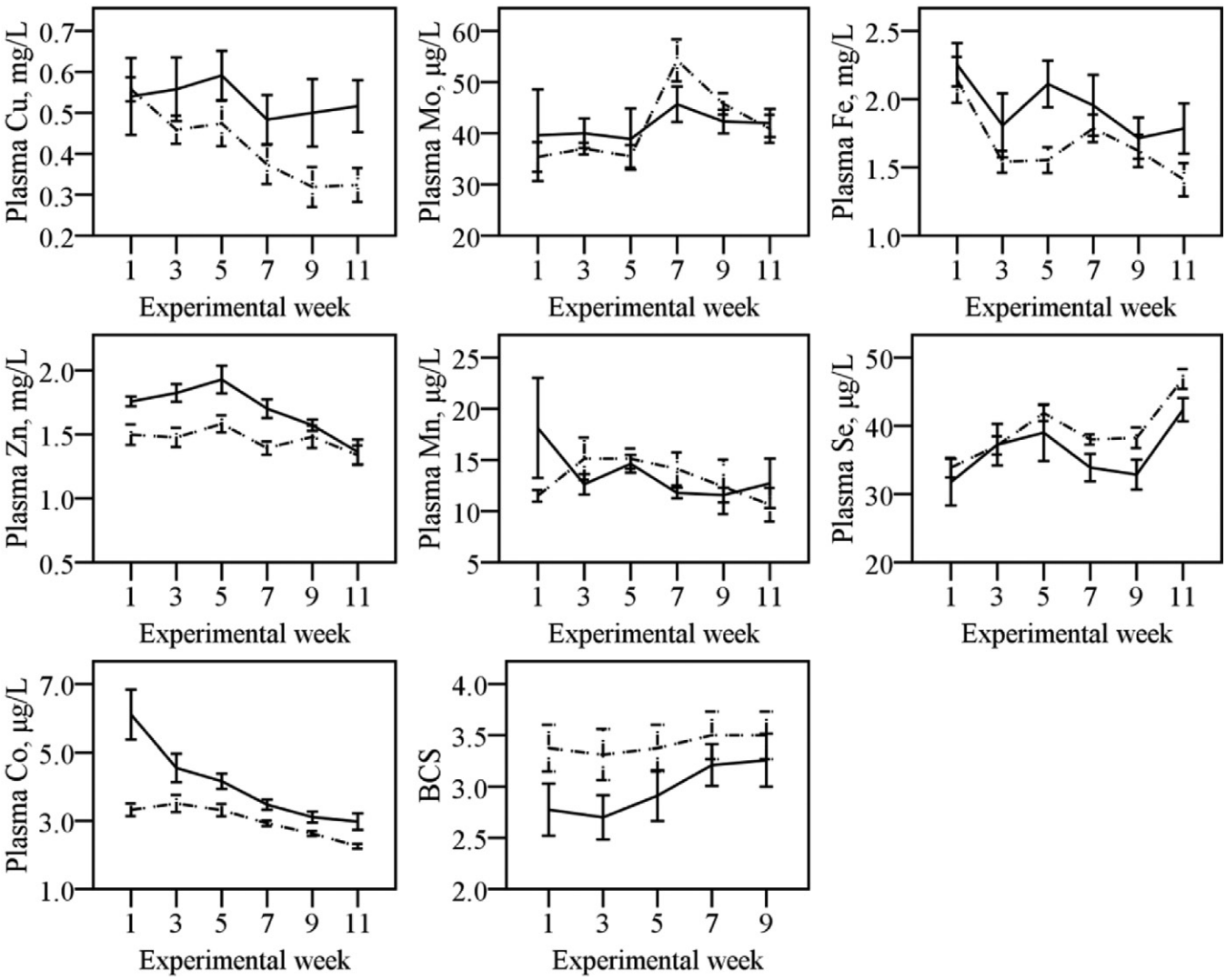

Figure 1. Plasma mineral concentrations and BCS in zebu (*Bos indicus*; $n = 8$) and crossbreed (*Bos indicus* × *Bos taurus*; $n = 8$) heifers on a Cu-deficient diet. Full lines represent zebu (*Bos indicus*) heifers, dotted lines represent crossed (*Bos indicus* × *Bos taurus*) heifers. Error bars represent ± SEM. There was a significant breed type × week interaction for Zn, Cu, Co, and BCS ($P < 0.05$), significant breed type effect for Zn and Co ($P < 0.05$), and significant time effect for all ($P < 0.05$) and a trend for Mn ($P = 0.05$).

(breed type × tissue; $P = 0.01$): Iron liver concentrations were greater in zebu than in crossbreed cattle ($P < 0.001$); other tissues were not impacted (cardiac muscle, $P = 0.9$; kidney, $P = 0.7$; and semitendinosus muscle, $P = 1.0$). For Zn and Mn concentrations, no differences in tissue concentrations were found between the 2 types of cattle (breed type × tissue; Zn, $P = 0.6$, and Mn, $P = 0.4$). Relative tissue Se concentrations differed in the 2 breed types (breed type × tissue; $P < 0.001$): zebu kidney Se concentrations were greater than crossbreed kidney Se concentrations ($P < 0.001$), but no differences were found for other tissues (cardiac and semitendinosus muscle, $P = 0.7$, and liver, $P = 0.4$). Finally, relative tissue Co concentrations were also affected by breed type (breed type × tissue; $P < 0.001$) and both cardiac muscle and kidney Co concentrations were greater in zebu than in crossbreed heifers ($P < 0.001$).

Tissue Gene Expression

For Cu regulatory genes, no significant type differences in relative expression of duodenal *Mt1a* mRNA were found ($P = 0.2$; Table 3). However, hepatic mRNA expression of Cu regulatory genes *Ctr1* ($P = 0.025$), *Cox17* ($P = 0.009$), *Atp7b* ($P = 0.022$), and *CCS* ($P = 0.029$) as well as *Sod1* ($P = 0.001$) were greater in zebu than in crossbred cattle. Relative mRNA expression of genes related to Cu function, duodenal *DAO*, and aorta *LOX* were not affected by type ($P = 0.8$). Breed type did affect relative mRNA expression of the Se-related *Gpx1* in liver ($P < 0.001$).

Relative mRNA expression of *Mt1a* and *CCS* were correlated with liver Cu concentrations, whereas liver Mo, Co and Mn concentrations were related to duodenal *DAO* mRNA expression. Kidney Se concentrations were positively associated with liver mRNA expression

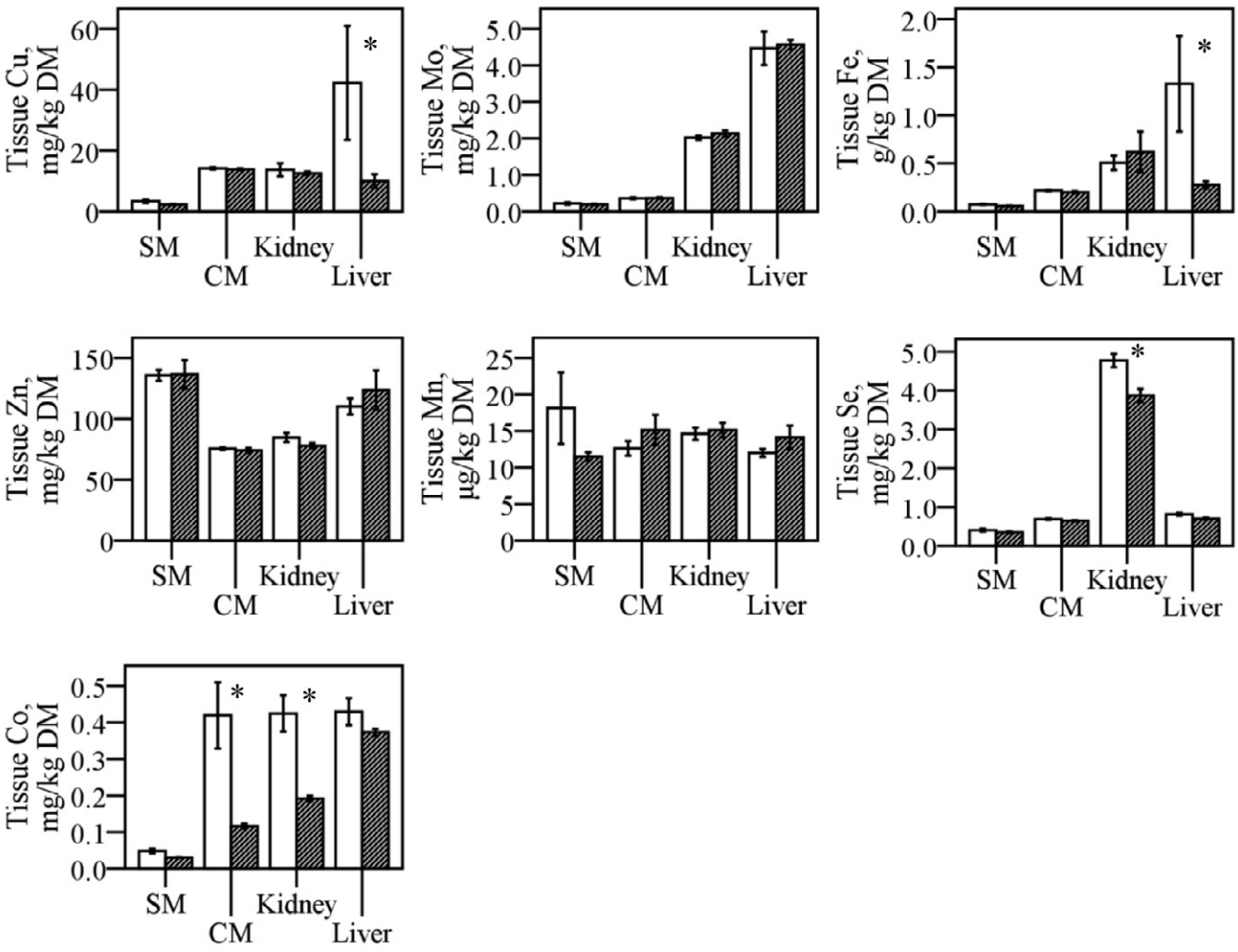

Figure 2. Tissue (SM = semitendinosus muscle, CM = cardiac muscle, trace element concentrations in zebu (*Bos indicus*; $n = 8$) and crossbreed (*Bos indicus* × *Bos taurus*; $n = 8$) heifers on a Cu-deficient diet. Clear bars represent zebu (*Bos indicus*) heifers. Dark bars represent crossbreed (*Bos indicus* × *Bos taurus*) heifers. Error bars represent ± SEM. Asterisks represent significant difference in tissue trace element concentrations between breed types of cattle ($P < 0.05$.

of *Atp7b*, *CCS*, *Cox17*, *Ctr1*, *Sod1*, and *Gpx1* (all $r > 0.5$; all $P < 0.050$; Table 4).

Performance

Initial BW was lower in zebu than in crosses (91 ± 5.3 vs. 139 ± 12.9 kg; $P = 0.004$). Initial BCS did not differ between breed types; on average, crossed heifers had an initial BCS of 3.4 ± 0.23 whereas on average, zebu heifers had an initial BCS of 2.8 ± 0.25 on a scale of 1 to 5 ($P = 0.1$).

Average daily gain was similar for both breed types ($P = 0.3$), but when expressed as percentage of initial BW, zebu gained more than crosses ($P = 0.014$), although the latter displayed a greater absolute DMI ($P = 0.009$) but not as percentage of BW ($P = 0.433$). Concomitantly, G:F was greater in zebu than in crosses ($P = 0.014$; Table 5). The BCS also evolved more positively for zebu than for crossbreeds (breed type × week interaction; $P = 0.006$; Fig. 1).

DISCUSSION

In the present study, zebu and crossbred cattle were kept on a Cu-deficient diet supplemented with Mo over 11 wk to investigate the effect on plasma and tissue Cu and other trace elements and related mRNA expression. With the given dietary Mo and S concentrations and supplemented Mo, at the onset of the trial, the calculated absorption of Cu ($\mathbf{A_{Cu}}$) of the unsupplemented diet, using the formula of Suttle (1983) for grass diets, $A_{Cu} = 5.7 - 1.3\ S - 2.785\ \log_e Mo + 0.227\ (Mo \times S)$, was 3.0%, whereas when supplemented with 1 mg/kg Mo, A_{Cu} dropped to 1.8%, and when supplementation was raised to 2 mg/kg Mo, A_{Cu} finally decreased to 0.7%. These values in combination with the low dietary Cu concentrations inevitably lead to a very low Cu uptake from the diet, with rapidly depleting reserves in the body as a consequence. Dietary Cu forms complexes with sulfides and thiomolybdates (by interaction with Mo and S), thus rendering Cu unabsorbable. The thiomolybdates could also have been absorbed

Table 3. Relative mRNA expression of trace element related genes in tissues harvested from zebu (*Bos indicus*; $n = 8$) and crossbreed (*Bos indicus* × *Bos taurus*; $n = 8$) heifers on a Cu-deficient diet[1]

Tissue	Gene	Zebu	Crossbreed	SEM	*P*-value
Cu regulatory genes					
Duodenum	*Mt1a*	29	8	8.2	0.23
Liver	*Ctr1*	3.7	2.0	0.39	0.03
	CCS	5.0	2.6	0.57	0.03
	Cox17	2.5	1.5	0.20	0.01
	Sod1	3.4	1.6	0.31	0.001
	Atp7b	1.9	1.3	0.14	0.02
Cu-related genes					
Aorta	*LOX*	2.5	2.4	0.29	0.84
Duodenum	*DAO*	9.1	10.3	1.89	0.77
Se-related gene					
Liver	*Gpx1*	3.0	1.5	0.24	<0.001

[1]Values are the mean relative copy number for the group.

through the ruminal wall, thereafter systemically binding Cu and causing a real thiomolybdate toxicity rather than a Cu deficiency (Gould and Kendall, 2011). However, the depletion of Cu reserves was reflected in the decreasing plasma Cu concentrations over time and low liver Cu concentrations at the end of the trial (overall mean = 26 mg Cu/kg DM). The Mo concentrations in plasma, on the other hand, were slightly increased over time as seen by Ivan and Veira (1985); although most of the Mo will be excreted in the form of the thiomolybdates, some may also be absorbed as MoO_4 (Ferguson et al., 1943). Concerning S, unfortunately, there is still no practical tool to evaluate the S status of cattle, as pointed out by Dermauw et al. (2012), and it was therefore not possible to investigate the evolution of this status over time.

Overall, a Cu deficiency caused by Mo and S interaction was evident. However, the response to the causative diet differed distinctly between the 2 breed types of cattle. Although plasma Cu concentrations were quite similar at the onset of the trial, over time they slowly decreased in *B. indicus* cattle to concentrations just below the threshold value for deficiency in *B. taurus* cattle (<0.57 mg Cu/L; Suttle, 2010), whereas *B. taurus* × *B. indicus* crosses developed extremely low plasma Cu concentrations. Furthermore, liver Cu concentrations were greater, yet also more variable, in zebu than in crossbred cattle (42 vs. 10 mg Cu/kg DM), 19 mg Cu/kg DM being the threshold for deficiency in *B. taurus* cattle (Suttle, 2010). Seemingly, crosses were more prone to Cu deficiency than zebu cattle. Miranda et al. (2010) suggested 4 different reasons for differences between cattle breeds in sensitivity towards Cu deficiency: differences in efficiency of absorption, differences in distribution among tissues, differences in excretion, or differences in feed intake. Concerning the latter, in the present study, we saw a similar DMI (% of BW) accompanied by a lower weight gain (% initial weight) in crossbred cattle. Therefore, the hypothesis of a greater feed intake does not seem to match with the results of our study.

Previous studies of breed sensitivity to Cu deficiency found differences between Angus and Simmental cattle, with much lower plasma Cu concentrations in Simmental cattle (Smart and Christensen, 1985). This was further investigated by Gooneratne et al. (1994), who found that Simmental cattle had a much greater biliary excretion of Cu. In the current study, we did not investigate differences in Cu excretion. Ward et al. (1995) showed that the Simmental breed seemed to have a lower apparent Cu absorption and Cu retention. Subsequently, Hansen et al. (2009) detected that Cu deficiency reduced mRNA expression of hepatic *Sod1*, whereas Fry et al. (2009) found decreased mRNA expression of *Cox17* and *Atp7b* and Hepburn et al. (2009) increased expression of *CCS* mRNA in Cu-deficient cattle. Fry et al. (2013) investigated differences in expression of Cu chaperones and transporters between Angus and Simmental cattle and detected a lower expression of duodenal copper transporters *Ctr1* and tendency for less *Atp7a* in Simmental cattle, suggesting a lower ability in these cattle to absorb and utilize dietary Cu. However, they did not detect differences in hepatic mRNA expression levels.

Table 4. Correlation coefficients between trace element storage and relative mRNA expression of trace element related genes in tissues harvested from zebu (*Bos indicus*; $n = 8$) and crossbreed (*Bos indicus* × *Bos taurus*; $n = 8$) heifers on a Cu-deficient diet

	Aorta	Intestine		Liver					
Parameter	*LOX*	*DAO*	*Mt1a*	*Atp7b*	*CCS*	*Cox17*	*Ctr1*	*Sod1*	*Gpx1*
Liver Cu	0.12	0.38	0.60*	0.07	0.58*	0.17	–0.13	0.15	0.22
Liver Mo	–0.27	0.65**	–0.21	–0.26	–0.29	–0.39	–0.32	–0.28	–0.33
Liver Fe	–0.06	0.33	0.15	0.36	0.23	0.13	0.15	0.28	0.33
Liver Mn	0.01	0.58*	0.03	0.09	0.17	0.06	0.01	0.23	0.17
Liver Zn	–0.06	–0.26	0.02	–0.07	–0.12	–0.10	–0.06	–0.15	–0.17
Kidney Se	0.22	–0.09	–0.20	0.57*	0.64**	0.55*	0.59*	0.73**	0.84**
Liver Co	0.09	0.53*	0.43	0.25	0.26	0.29	0.27	0.39	0.28

*$P < 0.05$; **$P < 0.01$.

In our study, where the induced Cu deficiency was more severe than in the study of Fry et al. (2013), zebu cattle seemed to have greater relative expression of the hepatic Cu transporters and chaperones *CCS*, *Ctr1*, *Cox17*, *Sod1*, and *Atp7b* mRNA. We did not investigate the intestinal expression of these genes, but zebu and crossbred cattle may also differ in expression of intestinal Cu transporters and chaperones, which could point to better absorption mechanisms in zebu vs. crossbred cattle, with greater liver and plasma Cu levels as a consequence. Although high variability in *Mt1a* mRNA expression did not allow discerning the potential differences between breed types, the positive correlation with liver Cu concentrations does point in this direction. Further research is necessary to confirm this. The expression of *CCS* was also positively associated with liver Cu, in contradiction with earlier data from Han et al. (2009). At this point, we could hypothesize that the greater expression of the hepatic Cu transporters and chaperones suggest that the zebu cattle have a greater Cu uptake in hepatocyte (*Ctr1*), combined with a greater Cu circulation (*CCS*) and incorporation in ceruloplasmin (*Atp7b*), and cytochrome c oxidase (*Cox17*), used for scavenging of superoxide ions (*Sod1*) within the hepatocyte as well as greater Cu excretion from the hepatocyte (*Atp7b*; Prohaska, 2004; Fry et al., 2013).

An overload of Fe can exacerbate a Cu deficiency, through exchanges of Fe sulfides with Cu to unabsorbable Cu sulfides or through formation of an equally insoluble Fe-Cu-S complex (Gould and Kendall, 2011). In the present study, Fe concentrations in the diet were not as high as previously found values in grasses in the same region (619 to 2,082 mg Fe/kg DM; Dermauw et al., 2013b), and the critical dietary Fe:Cu ratio was not reached (50 to 100; Suttle, 2010), so an additive effect of Fe on the Cu deficiency was not to be expected. On the contrary, the Cu deficiency seemed to have affected the Fe metabolism, which was reflected in our study by decreasing, but not deficient, plasma Fe concentrations over time and high liver Fe concentrations (overall mean = 801 mg Fe/kg DM). Hansen et al. (2010, p. 280) postulated that "Limited ceruloplasmin activity probably prevented Fe from being mobilized out of the liver, causing Fe to accumulate in the liver and limiting Fe availability for extrahepatic tissues," which may explain the decreasing plasma Fe concentrations noted in our study. The evolution of plasma Fe concentration over time did not differ between the 2 breed types of cattle. However, zebu cattle did have more variable and greater liver Fe concentrations than crossbred cattle, which were also greater than found in previous research in the area (Dermauw et al., 2013a). Although this difference seems to contradict the ceruloplasmin explanation of Hansen et al. (2010), dietary Cu levels were lower than in the supplemented study group of Hansen et al. (2010), and therefore, the range in Cu status is much smaller in our study. Nevertheless, the extremely high liver Fe concentrations in zebu cattle were striking and further research is warranted to determine the physiological reasons for this.

Table 5. Performance in zebu (*Bos indicus*; $n = 8$) and crossbreed (*Bos indicus* × *Bos taurus*; $n = 8$) heifers on a Cu-deficient diet

Parameter	Zebu	Crossbreed	SEM	*P*-value
Final BW, kg	103	148	9.4	0.01
ADG, kg	0.16	0.12	0.018	0.32
DMI, kg/d	1.92	2.82	0.184	0.01
G:F	0.08	0.04	0.009	0.01
DMI, % BW	1.98	1.98	0.003	0.51
Total gain, % initial BW	13	6.0	1.48	0.01

In the current study, zebu cattle had greater kidney Se concentrations than crosses and *Gpx*-1 mRNA expression was greater in zebu than in crosses, but no breed type × week interaction affected plasma Se concentrations. Langlands et al. (1980) previously found greater whole blood Se and Gpx1-Se activity in *B. indicus* (Afrikaander) than in *B. taurus* (Hereford-Shorthorn) cattle, with crosses having intermediate values. We know of no other studies investigating breed differences in bovine blood Se status or in kidney Se concentrations or *Gpx*1 mRNA expression. We do know that Cu and Se metabolism are interrelated, with lower Gpx1 activity and *Gpx*1 mRNA expression and greater fecal excretion of Se in Cu-deficient rats (Jenkinson et al., 1982; Olin et al., 1994). In this respect, the seemingly lower ability in crosses to cope with this Cu deficiency might have caused the lower mRNA expression of *Gpx1*. The positive association of Se storage and mRNA expression of Cu regulatory genes, *Ctr1*, *CCS*, *Atp7b*, *Cox17*, and *Sod1*, suggests shared pathways between Cu and Se yet to be elucidated. Overall, Se concentrations increased throughout the trial, which contradict the greater fecal excretion in Cu deficiency, but the rise, although significantly different over time, is small and may not be relevant. Finally, the differences between cattle breed types in plasma Co and semitendinosus muscle and kidney Co concentrations may be irrespective of the Cu deficiency but warrant further research on differences in trace element metabolism between *B. indicus* and *B. taurus* breed types of cattle.

In conclusion, *B. indicus* and *B. taurus* × *B. indicus* cattle had a disparate response to a Cu-deficient diet supplemented with Mo. Concentrations of Cu, both in transport and storage pools, were greater in *B. indicus* cattle than in crossbred cattle. In *B. indicus* cattle, this coincided with a greater mRNA expression of Cu regulatory and related genes in the liver. This may suggest a more efficient use of dietary Cu in *B. indicus* and a lower proneness to Cu deficiency in comparison with *B. taurus* × *B. indicus*

cattle. In *B. indicus* cattle, concentrations of Zn and Co in the transport pool and concentrations of Fe, Se, and Co in certain storage pools as well as a hepatic mRNA expression of the Se-related *Gpx1* were also observed to be greater. Overall, future research is warranted to fully unravel these potential differences in trace element metabolism between *B. indicus* and *B. taurus* × *B. indicus* cattle and to investigate to which extent such differences may also translate in different trace element requirements in the 2 breed types.

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